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13. ABSTRACT (Maximum 200 words)

The aims of this research are to study each of the various molecular mechanisms whereby toxic metal cations and oxyanions are chemically transformed by *Pseudomonas maltophilia* strain OR-02. The research effort for the current year has focused on the microbial-dependent transformations of mercury, selenium, tellurium, chromium and lead. The NADPH-dependent reduction of Hg(II) was catalyzed by an inducible mercuric reductase. The reduction of selenite and tellurite to their insoluble elemental forms was mediated by an intracellular glutathione reductase that utilized the spontaneously-formed bis(glutathio)Se(II) or bis(glutathio)Te(II), respectively, as pseudosubstrates. The 3-electron reduction of hexavalent chromium was catalyzed by a membrane-bound chromate reductase. The enzymatic basis for the transformation and immobilization of soluble lead(II) was not immediately apparent. This project could provide useful information toward the eventual exploitation of *P. maltophilia* and related organisms for the removal of toxic metal wastes from selected, heavily polluted sites.

14. SUBJECT TERMS
Bioremediation, Mercury, Selenium, Chromate, Lead

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A. RESEARCH OBJECTIVES

The aims of this project are to study each of the various molecular mechanisms whereby toxic metal cations and oxyanions are chemically transformed by a remarkable strain of <u>Pseudomonas maltophilia</u> originally isolated from mercury-contaminated soil at Oak Ridge National Laboratory. The specific aims for the current grant period are as follows:

(1) To perform detailed kinetic studies on selected metal transformations using suspensions of intact bacterial cells;

(2) To determine whether each metal transformation is a function of the bacterial cell itself or some exported component(s); and

(3) To identify, separate, purify, and reconstitute the minimum cellular components necessary for metal transformation.

The metal cations and oxyanions to be examined in these investigations include, but are not limited to, Se(IV), Cr(VI), Pb(II), Ag(I), Au(III), Cd(II), Sn(II) and Hg(II).

B. STATUS OF THE RESEARCH EFFORT

1. Specific Aim #1

When <u>P</u>. <u>maltophilia</u> strain O-2 was cultured in the presence of each of 8 different soluble metal species, growth of the organism was accompanied by the disappearance of the soluble metal species from solution, along with the concomitant appearance of an insoluble form of the metal. The kinetic properties of the <u>P</u>. <u>maltophilia</u>-dependent removal of soluble metals are shown in Fig. 1. Each curve in Fig. 1 was generated by the introduction of

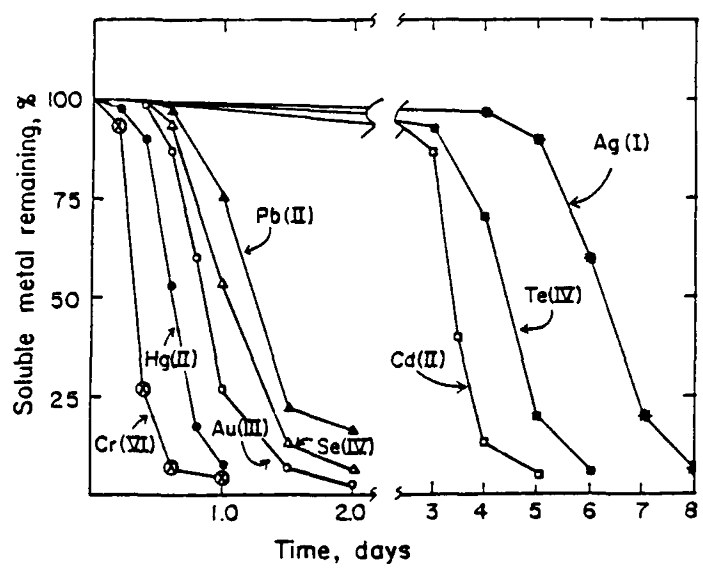

Fig. 1. Time courses of metal disappearance

naive, unadapted bacteria to the culture vessel containing the soluble metal species. Initial soluble metal concentrations in Fig. 1 were as follows: Cr(VI), 1.0 mM; Hg(II), 0.2 mM; Au(III), 3.0 mM; Se(IV), 40 mM; Pb(II), 3.0 mM; Cd(II), 3.0 mM; Te(IV), 10 mM; and Ag(I), 4.0 mM. The disappearances of 5 of the soluble metals in Fig. 1 were linked to biological reduction reactions. Selenite, tellurite, mercuric ions and chloroauric acid were each reduced to their respective elemental forms. The bacterial-dependent reduction of hexavalent chromium resulted in the transient formation of trivalent chromium, which subsequently disappeared from solution concomitantly with the formation of a white precipitate. Growth of strain O-2 in the presence of lead, cadmium, and silver ions resulted in the formation of brown-black, white, and gray-black precipitates, respectively, along with the concomitant removal of each soluble cation from solution.

Efforts to quantify the soluble metals featured in Fig. 1 were limited to UV-visible spectrophotometric assays that employed colorimetric reagents whose molecular absorption properties changed in the presence of specific metal ions. The practical detection limits of such assays are no lower than about 1.0 micromolar at best. It is anticipated that the impending acquisition of a combination flame/graphite furnace atomic absorption spectrophotometer (funded by the AFOSR) will lower the operational detection limit for most soluble metals shown in Fig. 1 by some 3 orders of magnitude. It will be of interest to investigate the kinetic properties of metal removal by strain O-2 at much lower concentrations of soluble metal than has heretofore been possible. While cell growth and metal-transformation activities at the high metal concentrations shown in Fig. 1 are unexpected and dramatic, it is the performance of metal-immobilization microbes at the lower concentrations more frequently encountered in polluted waste waters that is of the greater practical significance.

2. Specific Aim #2

The location, either intracellular or extracellular, of each metal precipitation activity attributed to strain O-2 was determined by scanning electron microscopic (SEM) and energy dispersive X-ray (EDX) analyses performed in collaboration with Drs. Larry Barton and Thomas Zocco at New Mexico State University and Los Alamos National Laboratory, respectively. Examination by SEM of cultures of O-2 grown in the presence of 40 mM selenite revealed 3 principal features: (i) intact bacterial cells frequently contained one or more electron dense bodies; (ii) other electron dense bodies were observed outside the cells; and (iii) numerous lysed cells and cell fragments were evident. EDX analyses on the same samples revealed that the electron dense bodies both inside and outside the cells were comprised exclusively of selenium and that these bodies accounted for all of the selenium in the field of vision. When O-2 was cultured in the presence of much lower concentrations of selenite, such as 1.0 mM or less, the number of both the extracellular selenium deposits and the lysed cells decreased dramatically relative to the number of cells with intracellular selenium deposits. On the

Table I. SEM/EDX analyses of metal precipitation by strain O-2

Soluble metal species	Intracellular ppt.	Extracellular ppt.
Se(IV)	X	
Te(IV)	X	
Hg(II)	X	
Au(III)		X
Ag(I)		X
Pb(II)		X
Cd(II)	Could not be determined	

hypothesis that the extracellular selenium deposits arose as a consequence of the lysis of bacterial cells at high selenite concentrations, the SEM and EDX analyses suggested that elemental Se^0 was produced intracellularly.

In contrast to the results obtained with selenite, growth of strain O-2 in the presence of lead nitrate led to the appearance of dark, electron dense bodies outside the bacterial cells. These dark extracellular bodies contained all of the detectable lead; lead could not be detected by EDX spectroscopy either inside the cell or on the plasma membrane. The SEM and EDX analyses thus indicated that insoluble lead was generated extracellularly.

Experiments such as those outlined above were conducted on each of 7 soluble metal species transformed and precipitated by strain O-2. The conclusions regarding the site(s) of metal transformation by strain O-2 are summarized in Table I, above.

3. Specific Aim #3

3a. Mercury - A mercuric reductase was purified to electrophoretic homogeneity from cell-free extracts of O-2 grown in the presence of 100 μM Hg(II). The purified enzyme was a soluble dimer comprised of identical subunits of 60,000 daltons. The enzyme contained one FAD per subunit and catalyzed the NADPH-dependent reduction of Hg(II) to Hg(0) in the presence of excess exogenous thiols. With 2-mercaptoethanol as the exogenous thiol, the reduction of mercuric ions obeyed Michaelis-Menten saturation kinetics with values of K_m for NADPH and RSHgSR of 15 and 6.0 μM, respectively, and a turnover number of 270 min^{-1}. The structural and functional properties of the mercuric reductase from O-2 were thus similar to those of analogous enzymes from other bacteria. The mercuric reductase-dependent oxidation of NADPH was entirely specific for mercuric ions. No enzyme-dependent oxidation of NADPH could be detected in the presence of any of the other 7 soluble metal species in Fig. 1.

3b. Selenium - Experimental results with both whole cells and cell-free extracts indicate that the bacterial-dependent generation of elemental selenium occurs as a consequence of the glutathione reductase-dependent reduction of the bis(glutathio)Se that forms spontaneously when selenite is exposed to a molar excess of reduced glutathione in the cytoplasm of the bacterial cell. A manuscript that describes these experiments with selenite is in preparation for submission to the Journal of Biological Chemistry. The manuscript has been written and awaits approval/revision by co-authors at other institutions prior to actual submission to the journal.

3c. Tellurium - The current working hypothesis is that the bacterial-dependent generation of elemental tellurium is strictly analogous to that of elemental selenium. That is, the reduction of tellurite to tellurium occurs as a consequence of the glutathione reductase-dependent reduction of the bis(glutathio)Te that forms spontaneously when tellurite is exposed to a molar excess of reduced glutathione in the cytoplasm of the bacterial cell. Rapid mixing spectrophotometric experiments to document and characterize the abiotic formation of bis(glutathio)Te are currently in progress. It is anticipated that these experiments will eventually lead to a separate publication.

3d. Chromium - Cells of strain O-2 that had been adapted for the reduction of chromate to chromium(III) were disrupted and examined for a cell-free chromate reductase activity. The only cell-free, pyridine nucleotide-dependent reduction of chromate that could be detected was located in the membrane fraction of cells that had been disrupted by *sonic oscillation*. Both NADH and NADPH supported chromate reduction. The pH optimum was 7.5 with an apparent K_m for chromate of 100 μM. The membrane-bound chromate reductase activity was quite labile and lost 70-80% of its original activity after 24 hours at 4° C. The enzyme(s) responsible for chromate reduction will not be investigated further at the present time because of this discouraging stability problem.

3e. Lead - The current goal remains the identification of the chemical nature of the brown-black precipitate generated when O-2 is grown in the presence of Pb(II). It is anticipated that IR spectrophotometry (performed in collaboration with Dr. John Henderson at Fisk University in Nashville, TN) may provide a definitive clue as to the biological agent(s) responsible for lead immobilization.

C. PUBLICATIONS

(i) Published - none

(ii) Submitted - one, "Chemical transformation of toxic metals by a Pseudomonas strain from a toxic waste site"; R.C. Blake, D.M. Choate, S.H. Bardhan, N.H. Revis, L.L. Barton, and T.G. Zocco; submitted to Environmental Toxicology and Chemistry

(iii) In preparation - one, "On the microbial-dependent transformation of toxic metals: mechanism of selenite reduction by *Pseudomonas maltophilia*"; R.C. Blake, D.M Choate, S.H. Bardhan, N.H. Revis, and J.H. Jackson; to be submitted to the *Journal of Biological Chemistry*

D. PROFESSIONAL PERSONNEL

(i) Postdoctoral Associate - None during the last year.

(ii) Research Assistant - Donna Choate, employed for the last 21 months

E. COUPLING ACTIVITIES

(i) Meeting presentations - two, "Chemical transformation of toxic metals by a *Pseudomonas* strain from a toxic waste site"; D. Choate, R.C. Blake, R. Revis; presented at the 1991 Annual Meeting of the American Society for Biochemistry and Molecular Biology held at Atlanta, GA; and

"Chemical transformation of toxic metals by a *Pseudomonas* strain from a toxic waste site"; R.C. Blake, D. Choate, and N.R. Revis; presented at the 11th Annual Meeting of the Society for Environmental Toxicology and Chemistry held at Washington, D.C.

(ii) Consultations - four invited seminars at the following institutions: New Mexico State University, Albuquerque, NM; Montana State University, Bozeman, MT; Los Alamos National Laboratory, Los Alamos, NM; and Chrysos, Inc., Tucson, AZ.

F. NEW DISCOVERIES

The discovery that strain O-2 will transform and precipitate soluble divalent platinum from solution.

G. OTHER STATEMENTS

The collaboration between this laboratory and that of Dr. Julius Jackson, a molecular geneticist at Michigan State University, has continued. Dr. Jackson has used plasmid DNA derived from strain O-2 to transform various metal-resistance phenotypes into a recipient strain of *E. coli*. He has supplied this laboratory with stable *E. coli* transformants that either (i) reduce Se(IV) to elemental selenium, (ii) reduce Hg(II) to elemental mercury, or (iii) immobilize Pb(II) as a brown-black precipitate. The acquisition of these transformants broadens the opportunities to study the molecular mechanism(s) of each metal transformation. The ability to transfer metal-immobilization phenotypes into other bacteria could also permit the eventual transformation of indigenous bacterial populations already adapted to and inhabiting selected heavily polluted sites.